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Significance of the Zero-Current Potential of the NAD^+ -NADH System *

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Summary

Since the first electrochemical reduction step of NAD^+ and the electrochemical oxidation step of NADH lie respectively at -0.69 and $0.5-0.9$ V (N.H.E.), no direct measure of the formal potential of NAD^+ -NADH system may be attained potentiometrically. We examine the significance of the previous potentiometric studies realized in the presence of a mediator and an enzyme. A reliable zero-current potential of NAD^+ and NADH solutions is only obtained when small amounts of benzyl-viologen (BV^{2+}) and xanthine oxidase (XO) are added. The various electrochemical reduction steps of 10 to 1000 μM BV^{2+} aqueous solutions are studied at pH 9.8 on dropping mercury and rotating platinum disk electrodes. The formal potential of the BV^{2+} - $\text{BV}^{\cdot+}$ system, equal to -0.360 V (N.H.E.), may be measured on a platinum electrode, while on a mercury electrode a strong adsorption interferes and gives a polarographic prewave. When XO is present, the addition of NADH decreases the BV^{2+} reduction waves: an anodic wave corresponding to $\text{BV}^{\cdot+}$ oxidation appears at the same potential as the first BV^{2+} reduction wave. The zero-current potential measured in NAD^+ , NADH, BV^{2+} , $\text{BV}^{\cdot+}$ and XO solutions is actually fixed by the BV^{2+} - $\text{BV}^{\cdot+}$ system, which equilibrates through chemical oxido-reduction reactions with the NAD^+ -NADH system.

Introduction

The pyridine coenzymes, such as NAD(P)^+ and NAD(P)H , are assumed to present well-known oxidation-reduction properties and, since the fifties, a formal potential of -0.32 V (N.H.E.)** has been assigned

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** All potential on referred to the Standard Hydrogen Electrode (S.H.E.).

to this redox system at pH 7. This value is commonly used for thermodynamical calculations and discussions, but few biochemists are aware of the way in which it was determined and of its significance.

The first estimates of the formal potential of this system were obtained from thermodynamical data relative to simple substrates and from equilibrium measurements.¹⁻⁵ The potential was calculated from the values of the equilibrium concentrations in mixtures of NAD^+ , NADH and, for example, ethanol and acetaldehyde in the presence of alcohol dehydrogenase.⁶ With such a method, one has to take into consideration the influence of the binding of the enzyme, if this catalyst is present in too high concentration: since the association constants of the enzyme with the oxidized and the reduced form of the coenzyme are generally different, a change in the concentration of the enzyme, may alter the proportion between the oxidized and the reduced free coenzyme and thus the equilibrium potential.⁷ In the case of alcohol dehydrogenase from horse liver and lactate dehydrogenase from beef heart, the potential of the enzyme-coenzyme complex system between pH 6 and 8 is 60–80 mV more positive than the potential of the free coenzyme.⁸

A qualitative estimate of the formal potential of the coenzyme is possible by observing whether reactions occur between the coenzyme and chemicals whose oxidation-reduction potentials are known from direct electrochemical measurements. In fact, only few such reactions are rapid enough in the absence of enzymes. The results obtained by this method are presented in Table 1 and show that the formal potential of the pyridine coenzymes or their N_1 alkyl models lies between -0.3 and -0.5V at pH 7.0.

The electrochemical reduction of aqueous solutions of pyridine nucleotides or their N_1 alkyl models has been extensively investigated by UNDERWOOD *et al.*,¹⁷⁻¹⁹ ELVING *et al.*,²⁰⁻²² and THÉVENOT *et al.*,²³⁻²⁵ The first reduction step of NAD(P)^+ or model compounds is reversible and pH-independent: it leads to the free radical NAD(P)^\bullet which dimerizes within a few milliseconds. The potential of the $\text{NAD(P)}^+ - \text{NAD(P)}^\bullet$ system, calculated from the half-wave potential and the dimerization rate constant is -0.82 V .^{25,26} The second reduction step of NAD(P)^+ or model compounds is irreversible and pH-dependent: it leads at least partially to NAD(P)H , but is difficult to observe because of the vicinity of the background discharge. The observed half-wave potential of $-1.3 \pm 0.2\text{ V}$ at pH 7 is considerably lower than the formal potential of the $\text{NAD(P)}^\bullet - \text{NAD(P)H}$ system calculated from the formal potentials of the $\text{NAD(P)}^+ - \text{NAD(P)}^\bullet$ and $\text{NAD(P)}^+ - \text{NAD(P)H}$ system: $U = [+0.47 - 0.06\text{ pH}]$ giving U' value at pH 7 of $+0.07\text{ V}$.²³

The electrochemical oxidation of aqueous solutions of pyridine nucleotides or their N_1 alkyl models has been more recently studied by LEDUC and THÉVENOT,^{11,12} ELVING *et al.*,²⁷ AIZAWA *et al.*,²⁸ and BLAEDEL and JENKINS.²⁹ All authors agree on the irreversibility of the single oxidation wave, leading to NAD(P)^+ or model compounds at a potential ranging between $+0.50$ and 0.95 V depending on the compound, the pH, the buffer, the electrode and the electrochemical method used. There are

Table 1. Chemical oxidation–reduction reactions involving NAD(P)⁺, NAD(P)H and N₁ alkyl nicotinamide models.

Electron donor	Electron acceptor	pH of experiment	Formal potential of the substrate V	Ref.
NMeNH	Ferricyanide	5.1 and 9.1	+0.41 (pH 4–13)	9, 10
NBzNH	id.	8.2	id.	11, 12
NMeNH	Benzoquinone	5.1 9.1	+0.40 (pH 5.1) +0.16 (pH 9.1)	10 10
NMeNH	2,6-dichloroindo-phenol	5.1 and 9.1	+0.22 (pH 7)	10
NCl ₂ BzNH	id.	7.0	id.	13
NBzNH	aniline–black suspensions	8.0	+0.20 (pH 3–13)	14
NMeNH	methylene blue	5.1 and 9.1	+0.01 (pH 7)	10
NBzNH	polypyrrole suspensions	8.0	–0.05 (pH 5–9)	14
NADH	2-amino 6,7-dihydro 6,7-dimethyl 4 (3H)pteridone (■)	8.8	–0.05 (pH 8.8)	15
NADH	safranine T		–0.29 (pH 7)	16
dithionite	NAD(P) ⁺ , NMeN ⁺ , NBzN ⁺	8–13	[–0.5 (pH 7) –0.7 (pH 9)]	8
2-amino 5,8-dihydro 7-methyl 4(3H)pteridone (■)	NAD ⁺	8.8	–0.65 (pH 8.8)	15
2-amino 5,8-dihydro 6,7-dimethyl 4(3H)pteridone (■)	NAD ⁺	8.8	–0.65 (pH 8.8)	15

Abbreviations: NMeNH, N₁-methyl-1,4-dihydro nicotinamide; NBzNH, N₁-benzyl-1,4-dihydro-nicotinamide; NCl₂BzNH, N₁-(2,6-dichloro benzyl)-1,4-dihydro-nicotinamide; NMeN⁺, N₁-methyl nicotinamide salt; NBzN⁺, N₁-benzyl nicotinamide salt.
(■) Electrochemically prepared *in situ*.

discrepancies among the data about the influence of the pH upon the potential of this step: the values of $\Delta U_{1/2}/\Delta \text{pH}$ or $\Delta U_p/\Delta \text{pH}$ obtained were 0^{11,12} for N_1 alkyl models, and -11 to -18 ^{27,29} and $+35$ mV per pH unit²⁸ for NAD(P)H in the 7–10 pH range.

The comparison between these electrochemical results and the data of Table 1, shows that both reduction of NAD(P)⁺ and oxidation of NAD(P)H can be much more easily achieved chemically than electrochemically. Thus direct electrochemical methods have been unable to bring the energetical and kinetical patterns of the transfer of two electrons and one proton to the condition occurring in chemical or biological reactions.

However, zero-current potential measurements were performed by RODKEY^{16,30} who studied mixtures of NAD(P)⁺ and NAD(P)H with a mediator, namely benzylviologen (BV^{2+}), and an enzyme, namely xanthine oxidase (XO). The potential values obtained decreased of 30 mV per pH unit; this is consistent with an exchange of two electrons and one proton, and was in very good agreement with the former equilibrium data. For this reason the value of -0.32 V was considered reliable and largely used since then.

In this paper we present electrochemical evidences for the interactions between NAD⁺, NADH, BV^{2+} and XO. It is shown that the existence of reliable zero-current potentials in mixtures of NAD⁺ and NADH is related to redox chemical reactions of the coenzyme with an electrochemical reversible system BV^{2+} – $\text{BV}^{\bullet+}$ catalysed by xanthine oxidase.

Experimental

Materials

Nicotinamide–adenine–dinucleotide (NAD⁺, purity 98 %), 1,4-dihydro–nicotinamide–adenine, dinucleotide (NADH, purity 98 %) (SIGMA CHEMICAL), benzyl viologen (BRITISH DRUG HOUSE) and rosinduline 2G (K. and K.) were used without further purification. Sturgeon D glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) (M.W. 145,000 daltons) was generously provided by F. SEYDOUX (University of Paris XI, Orsay) in a high purity holoenzyme form;³¹ the ammonium sulfate suspension was centrifugated, dissolved in ethylene diamine buffer at pH 7 and dialysed overnight to remove ammonium sulphate and the excess of NAD⁺. Milk xanthine oxidase (EC 1.2.3.2.) (M.W. 275,000 daltons) was obtained from BOEHRINGER (10 mg/cm³) in saturated ammonium sulfate and used without separation. Thus our main buffer, *i.e.* glycine–glycinate (MERCK, *pro analysi*), was used at high concentrations: 1 M for total glycine. The pH shifted from 9.8 to 9.5 when xanthine oxidase was added (2.5 μM final concentration) because of the presence of ammonium sulfate.

Electrodes

The working electrode for zero-current potential measurements was a platinum sheet of 0.5×0.5 cm. Our rotating electrode was a platinum disk with 2 mm diameter (TACUSSEL EDI, associated with a TACUSSEL Controvit rotation monitor). Reference electrodes were $\text{Ag}|\text{AgCl}$, saturated KCl (TACUSSEL AgCl 10). The counter electrode was the platinum sheet mentioned before.

Apparatus and procedure

Zero-current potentials were recorded on a TACUSSEL EPL 2 recorder with a TAT 4 plug-in unit. Polarographic and voltammetric studies were performed with the same recorder and with a TV 11 GD plug-in unit, a TACUSSEL PRT 20-2X potentiostat and a PRG 3 interface for *d.c.* and *a.c.* measurements. For drop-time controlled polarography we used a 30 cm capillary with a mercury height of 50 cm; the free drop-time was 8.2 s and the mercury flow rate 0.85 mg s^{-1} . The drop-time was controlled with a TACUSSEL MPO electrical hammer to a 0.5 s value.

All measurements were performed in a TACUSSEL MCT jacketed cell with a solution volume ranging from 2 to 5 cm^3 . It was thermostated at $30 \pm 0.2^\circ\text{C}$. All solutions were outgassed with a nitrogen stream which was deoxygenated by bubbling through a pyrogallate alkaline solution.

NAD^+ and NADH concentrations were checked with a CARY 14 recording U.V. spectrophotometer.

Voltammetric and polarographic studies were made with a scan rate of 2 mV/s. The rotation speed of the platinum disk electrode was usually 2,500 *r.p.m.* For *a.c.* measurements, the superimposed signal had generally a 10 mV amplitude and a frequency of 40 Hz for in-phase and 400 Hz for quadrature demodulation. *d.c.* voltammograms obtained with very diluted benzyl viologen solutions (20 μM) were subtracted from background curves before the determination of the characteristics of the waves.

Results and discussion

Zero-current potential measurements in NAD^+ -NADH mixtures

The electrochemical patterns of aqueous solutions of NAD^+ , NADH and N_1 -alkyl-nicotinamide model compounds (Fig. 1) show evidence that simple mixtures of 1 mM NAD^+ and 1 mM NADH cannot give well-defined zero-current potentials. In fact, the measured potential of such mixtures may drift at random for several tenth of volt.

Addition of mediators like benzyl-viologen or rosinduline 2G (in their oxidized form) decreases the potential but does not lead to an equilibrium potential. Likewise, addition of a specific dehydrogenase such as sturgeon D glyceraldehyde-3-phosphate-dehydrogenase (GPDH) (final

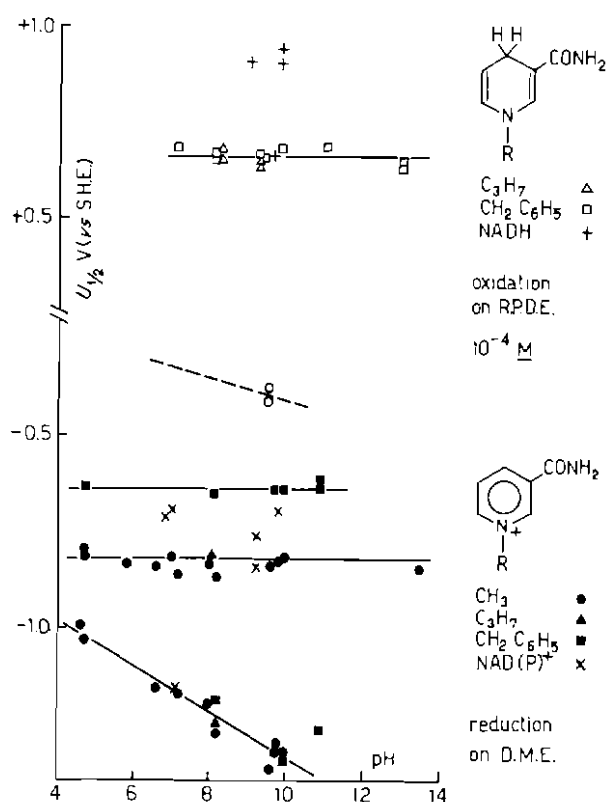


Fig. 1.
 $U_{1/2}$ vs. pH diagram for the polarographic reduction of 0.1 mM NAD(P)⁺ and N_1 alkyl model compounds aqueous solutions (lower part) and for the voltammetric oxidation of 0.1 mM NADH and N_1 alkyl model compounds aqueous solutions on a R.P.D.E. (higher part).^{12,25}
 (O) zero-current potential of a platinum electrode dipped into an aqueous solution containing 0.5 mM NAD⁺, 1 mM NADH, 20 μ M BV²⁺ and about 1 μ M XO, in glycine buffer at 30 °C.
 (—) Formal potential of the NAD(P)⁺ - NADPH system calculated from equilibrium concentration values in the presence of substrate and corresponding dehydrogenase.¹⁻⁸

concentration reaching 0.18 mM in monomer) or of less specific oxidases like milk xanthine oxidase (XO) (final concentration generally 1 μ M) does not stabilize the zero-current potential. Even addition of a mediator like benzyl-viologen to mixtures of NAD⁺, NADH and GPDH does not stop the potential drift.

On the other hand, when we replaced GPDH by XO in the former solution we obtained, as RODKEY¹⁶ did, stable and reproducible zero-current potentials. In a typical experiment, similar to those performed by RODKEY, a mixture of 0.5 mM NAD^+ , 1 mM NADH, 20 μM benzylviologen and about 0.25 mg/cm³ XO in glycine buffer at pH 9.5 gave at a platinum electrode an equilibrium potential of -0.408 ± 0.005 V within 20 min; this value corresponds to a formal potential at pH 7 of -0.324 ± 0.005 V which is in perfect agreement with the previous data. However, when we replace in the same solution the platinum working electrode with a D.M.E., a stable zero-current potential of -0.182 ± 0.005 V is obtained; this potential corresponds to the first reduction step of BV^{2+} on the mercury electrode as described below. This discrepancy of zero-current potentials shows that in such experiments there is not always an equilibrium between the electrode surface and the components of the solution. However, when 25 fold more concentrated BV^{2+} solutions *i.e.* 0.5 mM BV^{2+} , 1 mM NAD^+ , 12.5 mM NADH and 2.5 μM XO were used, we obtained a potential of -0.42 V at pH 9.5, both with D.M.E. and with Pt electrode. The equality of these zero-current potentials shows evidence of an equilibrium between the electrode surface and the species in solution. The resulting formal potential of NAD^+ -NADH at pH 7 is -0.321 ± 0.005 V. On the other hand, the use of glassy carbon or gold disk electrodes leads to a less stable and considerably less negative potential than those obtained with platinum and mercury electrodes (between -0.3 and $+0.1$ V).

Electrochemical reduction of benzyl-viologen (BV^{2+})

a - On a dropping mercury electrode.

d.c. and *a.c.* polarograms of BV^{2+} present different features according to its concentration value, compared to 0.2 mM.

In a 0.5 mM BV^{2+} solution buffered at pH 9.8 five cathodic *d.c.* waves, numbered I to V, are obtained: their $U_{1/2}$ are respectively -0.085 , -0.365 , -0.523 , -0.879 and -1.015 ± 0.010 V (Fig. 2). These steps are accompanied by five in-phase *a.c.* peaks whose U_p are respectively -0.085 , -0.349 , -0.526 , -0.874 and -1.028 ± 0.010 V. Steps I and V are accompanied by quadrature *a.c.* peaks whose U_p are respectively -0.090 and -1.028 ± 0.010 V.

Two additional *a.c.* peaks are present on both in-phase and quadrature polarograms at -0.720 and -0.935 ± 0.010 V and a quadrature *a.c.* peak is observed at -0.797 V.

In BV^{2+} solutions of concentration lower or equal to 0.2 mM, steps II and III are absent (Fig. 3); *d.c.* wave I is generally poorly defined unless the background current is not subtracted: if such a correction is achieved wave I resolves into two waves of $U_{1/2}$ -0.007 and -0.183 ± 0.010 V respectively, for a 20 μM BV^{2+} solution (Fig. 5).

Cyclic voltammograms of 0.5 mM BV^{2+} on a D.M.E. show two reversible steps followed by an irreversible one: for a sweep rate of 0.1 V/s the U_p of the cathodic peaks equals respectively -0.092 (I_c),

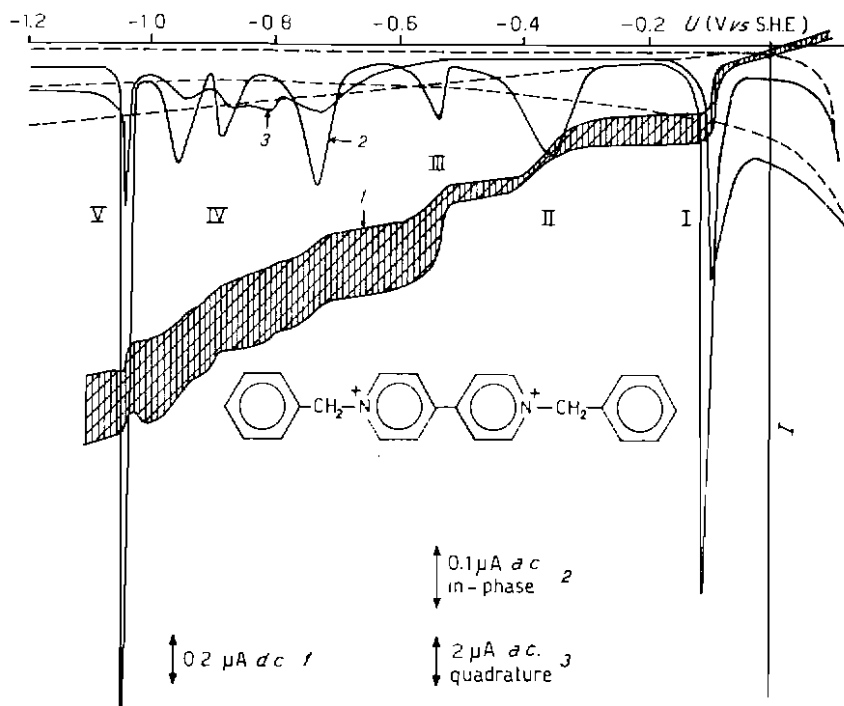


Fig. 2.
d.c. and a.c. polarograms of benzyl-viologen (BV^{2+}) 0.5 mM in glycine buffer (pH 9.8). Controlled drop-time 0.5 s, 30 °C. For a.c. polarograms, the superimposed signal had a 10 mV amplitude and a frequency of 40 Hz for in-phase and 400 Hz for quadrature demodulation. Dashed line is background electrolyte base current.

-0.376 (II_c) and -0.532 ± 0.005 V (III_c) while the U_p of the anodic peaks, obtained on return scan, equal respectively -0.088 (I_a), -0.324 (II_a) and -0.386 ± 0.005 V (III_a). Whereas peaks II_c and II_a present the usual features, peaks I_c and I_a are abnormally sharp and the current of peak I_a is at least 3 times larger than the one of peak I_c .

The potentials of d.c. wave I and a.c. peak I increase of about +65 mV per BV^{2+} concentration decade in the $10 \mu M - 1 mM$ range (Fig. 3). The height of d.c. wave I increases in proportion to the concentration till a limited value for BV^{2+} concentrations larger than 0.2 mM (Fig. 4) is reached; meanwhile, the heights of both in-phase and quadrature a.c. peak I are proportional to the concentration in the 0.01–1 mM range. This first reduction step is generally interpreted as a prewave corresponding to the adsorption of either the singly or the doubly reduced BV^{2+} i.e. $BV^{•+}$ or BV^0 , produced at the electrode;^{32,33} the mean adsorption area of this specie may be estimated from the limiting value the wave-height :

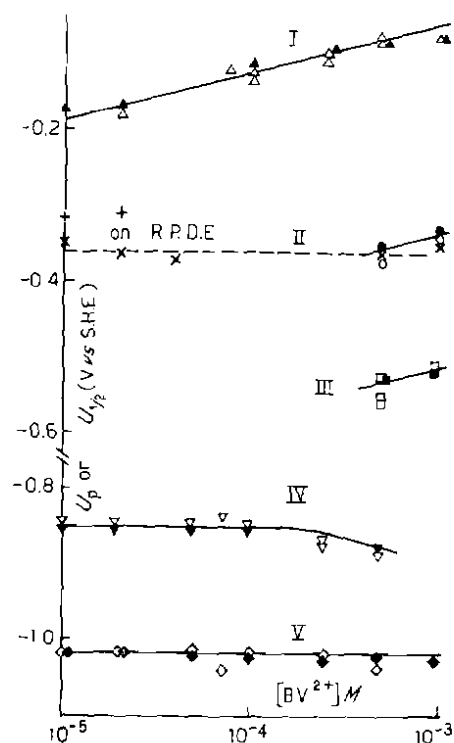


Fig. 3. Influence of benzyl-viologen concentration on the $U_{1/2}$ or U_p of its different reduction waves or peaks in glycine buffer (pH 9.8). (open points and \times) *d.c.* $U_{1/2}$; (full points and $+$) in-phase *a.c.* U_p ; (\times and $+$) R.P.D.E. (triangles, circles and squares) D.M.E.

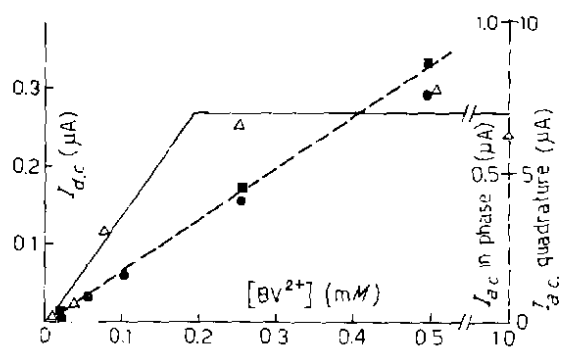


Fig. 4. Influence of benzyl-viologen concentration on the heights of its first *d.c.* wave and *a.c.* peaks in glycine buffer (pH 9.8). (Δ) direct current; (\blacksquare) in-phase and (\bullet) quadrature *a.c.* components, respective frequencies 40 and 400 Hz , $\Delta U = 10$ mV.

$A = 13.66 n m^{2/3} / (I_l \tau^{1/3}) = 57 \pm 7 \text{ \AA}^2$ where n is the number of electrons involved assumed here to be 1, m is expressed in mg/s, I_l in μA and τ , the controlled drop-time, in s. The adsorption area obtained is somewhat smaller than the value obtained with methylene blue³⁵ (100 \AA^2) and much larger than the value obtained with pyrimidine³⁶ ($3\text{--}7 \text{ \AA}^2$).

The potentials of *d.c.* waves and in-phase *a.c.* peaks II and III increase slightly with BV^{2+} concentration (Fig. 3) and have been reported to be pH-independent in the 1.1–10.0 range³². Sums of the heights of either *d.c.* waves I and II or I and III are proportional to BV^{2+} concentration. Step II, the potential of which is very close to the formal potential of $\text{BV}^{2+}\text{--BV}^{\cdot+}$, determined potentiometrically, *i.e.* -0.359 V ,³⁷ is generally interpreted as the formation of the blue free radical $\text{BV}^{\cdot+}$.³² Step III presents patterns similar to those of step II: it is reported to be related to the formation of an electron-inactive species, probably BV^0 .³²

Finally, *d.c.* waves and *a.c.* peaks IV and V present usual diffusion controlled features, for their potentials are concentration-independent (Fig. 3) and their heights are proportional to the BV^{2+} concentration, up to a 0.2 mM value. The $U_{1/2}$ and the wave heights of both waves have been reported to depend upon the pH.³² They may correspond to the third and fourth electron uptake of BV^{2+} although this has not been demonstrated.

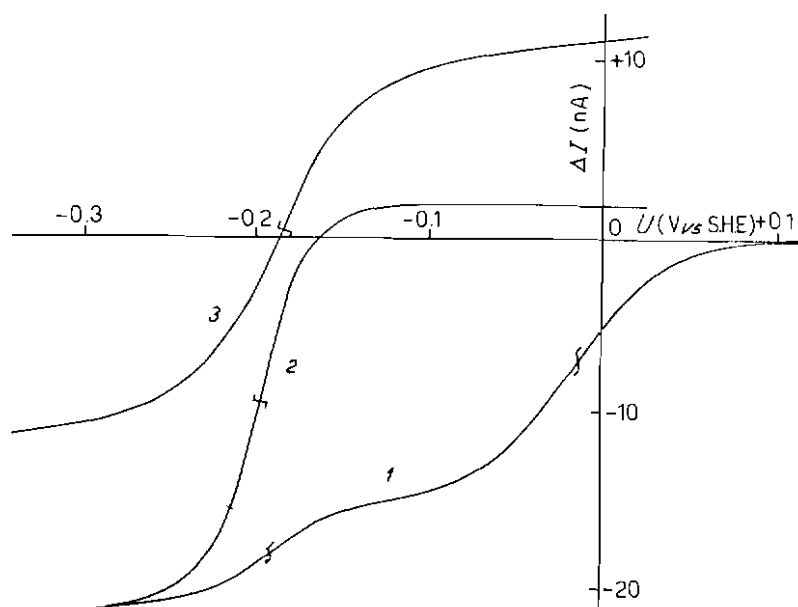


Fig. 5. Differential *d.c.* polarograms of benzyl viologen in glycine buffer D.M.E., pH 9.5. (1) $20 \mu\text{M}$ benzyl viologen, (2) as (1) with 0.5 mM NADH and (3) as (2) with $2.5 \mu\text{M}$ xanthine oxidase. Background base current was subtracted and each curve is the average of two independent experiments.

b - On platinum and gold disk electrodes

d.c. and *a.c.* current *vs.* potential curves obtained with BV^{2+} solutions on rotating platinum disk electrodes (R.P.D.E.) are less simple than the corresponding polarograms. A single cathodic *d.c.* wave, whose $U_{1/2}$ equals -0.360 ± 0.010 V in a $20\text{--}1000 \mu\text{M}$ BV^{2+} solution buffered at pH 9.8, is accompanied by both in-phase and quadrature peaks whose U_p are respectively -0.305 and -0.295 ± 0.010 V (Fig. 3 and 7). If the potential reaches -0.6 V, another group of in-phase and quadrature peaks may be obtained with U_p of -0.555 ± 0.010 V: under such conditions, the R.P.D.E. is covered with an adsorbed product of electrolysis which on return scan gives two anodic *d.c.* peaks (-0.32 and -0.37 V), two in-phase *a.c.* peaks (-0.32 and -0.38 V) and a quadrature *a.c.* peaks (-0.26 V). It is worth of note that the $U_{1/2}$ of the single *d.c.* wave on R.P.D.E. equals the formal potential of BV^{2+} - BV^{+} ³⁷ and the $U_{1/2}$ of the second *d.c.* polarographic wave (respectively -0.359 and -0.365 V).

Direct current *vs.* potential curves obtained on a rotating gold disk electrode are very similar to those obtained on R.P.D.E.: $U_{1/2}$ equals -0.353 ± 0.010 V in a 0.5 mM BV^{2+} solution buffered at pH 9.8.

Cyclic voltammograms of 0.5 mM BV^{2+} on a stationary platinum disk electrode shows a single reversible step at a potential higher than -0.5 V. For a sweep rate of 0.1 V/s , the U_p of the cathodic peak equals -0.38 ± 0.01 V and the U_p of the anodic peak, obtained on return scan equals -0.31 ± 0.01 V.

Electrochemical behaviour of mixtures of BV^{2+} , NADH and NAD^+ *a* - On a dropping mercury electrode

Addition of NADH or NAD^- , at the same concentration as BV^{2+} , shifts the BV^{2+} first reduction step (*d.c.* wave and *a.c.* peaks I) towards more negative potentials (maximum effect -50 mV); meanwhile the heights of *d.c.* wave and *a.c.* peaks I are slightly increased ($+20\text{--}30 \%$ depending on the BV^{2+} concentration) (Fig. 6). Addition of a large excess of NADH in very diluted BV^{2+} solution seems to enhance the slope of the BV^{2+} *d.c.* wave I: when the background current is subtracted, it appears clearly that the ill-defined prewave situated at -0.007 V disappears completely while the wave at -0.182 V increases so that total height is not modified (Fig. 5). On such background-subtracted polarograms it appears that a small part of wave I is now anodic: it means that with a 25 fold excess of NADH, BV^{2+} is very partially reduced into adsorbed BV^{+} even when an enzyme catalyst is absent. On a similar experiment achieved with more concentrated BV^{2+} and NADH solutions (Fig. 6) this effect is more noticeable; as the blue colour of BV^{+} does not appear in such solutions it is obvious that only adsorbed BV^{+} is produced.

No significant modification of the other reduction *d.c.* waves or *a.c.* peaks of BV^{2+} has been observed when equivalent or excess amounts of NADH are added.

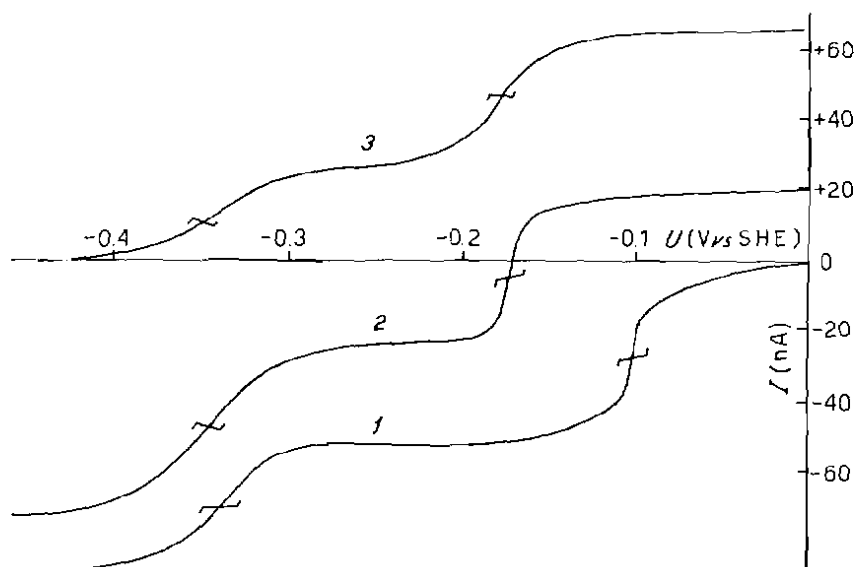


Fig. 6.
Influence of NADH, NAD⁺ and XO addition on the *d.c.* polarographic waves of 0.5 mM benzyl-viologen in glycine buffer (pH 9.5) (1) 0.5 mM BV²⁺, (2) as (1) with 12.5 mM NADH and 1 mM NAD⁺, (3) as (2) with 2.5 μM XO.

On the other hand, addition of BV²⁺ up to 20–500 μM does not modify the first reduction *d.c.* wave of a 0.5 mM NAD⁺ solution whose $U_{1/2}$ equals -0.657 ± 0.010 V at pH 9.8.

b – On platinum and gold disk electrodes

Addition of a mixture of either 1.2 mM NADH and 3 mM NAD⁺ or of 0.5 mM NADH (Fig. 7) does not modify the single *d.c.* reduction wave of a 40 μM BV²⁺ solution and its corresponding *a.c.* peaks.

On the other hand, addition of BV²⁺ up to 20–500 μM does not modify the single anodic *d.c.* wave of a 0.5 mM NADH solution buffered at pH 9.8 whose $U_{1/2}$ equals $+0.91 \pm 0.02$ V.

Electrochemical behaviour of mixtures of BV²⁺, NADH, NAD⁺ and xanthine oxidase

1–2.5 μA XO solutions buffered at pH 9.5 exhibit neither cathodic nor anodic *d.c.* wave or *a.c.* peak on D.M.E. nor R.P.D.E. when the same current scales are used. Added to 0.5 mM NADH or NAD⁺, such XO solutions do not modify appreciably their respective anodic or cathodic *d.c.* waves nor *a.c.* peaks. No significant modification of the various cath-

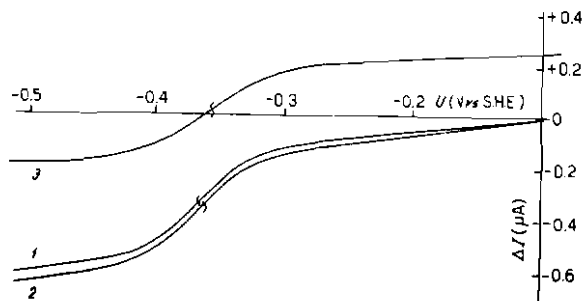


Fig. 7. Differential *d.c.* voltammograms of benzyl-viologen on R.P.D.E. in glycine buffer (pH 9.5). (1) $40 \mu\text{M}$ benzyl-viologen, (2) as (1) with 0.5 mM NADH and (3) as (2) with $1 \mu\text{M}$ xanthine oxidase. Background base current was subtracted.

odic steps of BV^{2+} on D.M.E. and R.P.D.E. is detected even when XO is added to the BV^{2+} solutions. As usually observed in protein solutions, the background discharge on D.M.E. is shifted towards less negative potentials when $1\text{--}2.5 \mu\text{M}$ XO is added.

However, in apparent contradiction with these stationary experiments, we have observed on cyclic voltammograms on D.M.E. that the U_p of 0.5 mM NAD^+ is shifted from -0.744 to $-0.904 \pm 0.005 \text{ V}$ when $2.5 \mu\text{M}$ XO is added; meanwhile the peak current increases of about 30 %. This is probably related to adsorption phenomena: indeed BURNETT and UNDERWOOD¹⁷ and ELVING *et al.*²² observed a potential shift of the NAD^+ first *d.c.* polarographic wave of respectively -190 and -140 mV when KCl is replaced by Bu_4NClO_4 and Et_4NCl in their supporting electrolyte. Thus, no further evidence of any association or chemical reaction between NAD^+ and XO results from such experiments.

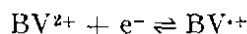
On the other hand addition of small amounts of XO changes considerably the reactivity of BV^{2+} towards NADH (blue coloured $\text{BV}^{\cdot+}$ appears) and the electrochemical behaviour of mixtures of BV^{2+} and NADH.

When $2.5 \mu\text{M}$ XO is added into a $20 \mu\text{M}$ BV^{2+} and 0.5 mM NADH solution the height of the cathodic polarographic wave I of BV^{2+} decreases, whereas its $U_{1/2}$ remains constant (Fig. 5). Meanwhile an anodic wave I_a appears at the same potential; its height reaches a limiting value almost equal to that of wave I; the sum of both anodic and cathodic wave heights is constant. A zero-current potential can be read on the curve, which is consistent with the direct measurement previously mentioned i.e. -0.182 V .

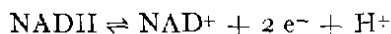
On the other hand in a solution containing BV^{2+} 0.5 mM , NAD^+ 1 mM , NADH 12.5 mM , addition of XO $2.5 \mu\text{M}$ causes the complete disappearance of wave I, this step becoming only anodic; meanwhile wave II also decreases and an anodic wave II_a appears at the same po-

tential, the sum of both cathodic and anodic wave heights decreasing of about 50 %. Current-potential curves cross the potential axis at -0.42 V which is the zero-current potential previously mentioned in such mixtures.

The behaviour of such NAD^+ , NADH , BV^{2+} , $\text{BV}^{•+}$ and XO solutions on D.M.E. may be interpreted in different ways according to the BV^{2+} and $\text{BV}^{•+}$ concentration. For very diluted dye solutions it seems that we face probably an adsorption of $\text{BV}^{•+}$ which generates prewave I; there is no equilibrium between the adsorbed species and the solution, as demonstrated by the difference of zero-current potentials on D.M.E. and on platinum electrode: apparently the adsorption equilibrium is not yet reached when the mercury drop falls and the electrode surface is renewed (0.5 s). When the dye concentration is large enough, namely of more than 0.2 mM, the cathodic wave is not present any more and the anodic waves I_a and II_a appear; thus the step which gives rise to a zero-current potential on D.M.E. is now step II



which equilibrates with the oxidation reaction in the bulk of the solution



A similar electrochemical behaviour of dyes was described by MÜLLER,³⁸ for example by progressive reduction of rosinduline 2G by hydrogen gas in the presence of platinized asbestos.

With a $20 \mu\text{M}$ BV^{2+} , 0.5 mM NADH and $1 \mu\text{M}$ XO solution we obtained on a rotating gold disk electrode an ill-defined cathodic wave corresponding to the reduction of BV^{2+} and no anodic wave corresponding to the oxidation of $\text{BV}^{•+}$, the presence of which is demonstrated by the blue colour of the solution. The potential of the cathodic wave is the same as the one observed without xanthine oxidase but the height is 10 times smaller. The zero-current potential obtained with such experiments is ill-defined, for the I vs. U curve crosses the potential axis with a very slight slope; its value is about -0.2 V. Apparently there is no diffusion of $\text{BV}^{•+}$ from the solution to the electrode surface; this behaviour is probably due to a fouling of the gold electrode by xanthine oxidase.

On R.P.D.E., addition of XO up to about $1 \mu\text{M}$ into a $40 \mu\text{M}$ BV^{2+} and 0.5 mM NADH solution decreases the BV^{2+} cathodic wave to about half of its original height while a new anodic wave of equal height appears at the same potential (Fig. 7).

The interpretation of our R.P.D.E. experiments is easier than that of the D.M.E. ones, as no adsorption phenomena interfere with the BV^{2+} reduction. It is clear that the anodic wave, appearing mainly when XO is added, is related to $\text{BV}^{•+}$ oxidation. Thus BV^{2+} and $\text{BV}^{•+}$ concentrations at equilibrium may be estimated by the height of the cathodic and anodic *d.c.* wave respectively. In the experiments described in Fig. 7 unknown quantities of NAD^+ were added as impurities of NADH and/or as the product of oxidation of NADH by traces of oxygen (this reaction

is also catalysed by XO). This is why, in another set of experiments, we mixed 1.3 mM NADH, 3 mM NAD⁺, 20 μ M BV²⁺ and about 1 μ M XO; the apparent equilibrium constant

$$K_{app.} = \frac{[NAD^+][BV^{•+}]^2}{[NADH][BV^{2+}]^2}$$

was estimated to be 18.4, whereas, using the formal potentials, at pH 7, –0.320 and –0.360 V, respectively, for NAD⁺–NADH and BV²⁺–BV^{•+}, the calculated theoretical value, is 15.8 at pH 9.5. The agreement between these two values of $K_{app.}$ is good when the estimated prediction of the various concentrations is taken into account.

Conclusions

The impossibility of measuring a stable zero-current potential of solutions containing only NAD⁺ and NADH is not surprising when one considers the electrochemical data obtained with both compounds and models, summarized in Fig. 1: a difference of at least 1.3 V lies between the $U_{1/2}$ of their first reduction and oxidation steps. It is clear that a definite and stable zero-current potential may be obtained only if an electrochemically reversible system is present in the solution. Two ways may be considered to fulfil this condition and to measure the formal potential of the NAD⁺–NADH system.

1. One may modify the electrochemical irreversible NAD⁺–NADH system by using a catalyst, which changes the kinetics and/or the mechanism of the electrochemical reduction and oxidation of the coenzyme, thus originating a reversible two electrons — one proton step, as is assumed to occur *in vivo*. If the electrochemical reversible system is for example NAD, X–NADH, X the two associations between ligand X (the catalyst) and the oxidized coenzyme on one hand and the reduced coenzyme on the other should be taken into account in the determination of the formal potential of the NAD⁺–NADH system. Such an hypothesis does not seem unreasonable, as it has been reported that, *in vivo*, the pyridine coenzymes are probably entirely associated to various dehydrogenases and oxido-reductases.⁸ We have tried to prove such a catalytic effect by different electrochemical methods *i.e.* voltammetry on R.P.D.E., polarography and zero-current potentiometry on platinum, vitreous carbon and hanging mercury electrode. All attempts carried out with dyes such as BV²⁺ or rosinduline 2G and/or sturgeon GPDH or milk XO were unsuccessful. Such a catalytic effect was not observed by ITO and KUWANA³⁹ either on voltammetric curves performed with tin oxide optically transparent electrodes and solutions containing 1 mM NADP⁺, 0.4 mM methyl viologen and 0.43 mg/cm³ spinach ferredoxin–NADP–reductase buffered at pH 7.0.

2. One can alternatively replace the electrochemical irreversible

NAD⁺-NADH system by a reversible one, which would equilibrate with it by a rapid chemical reaction. Such a reaction has been reported¹⁰ between N₁-methylnicotinamide models of NAD(H) and rosininduline 2G: corresponding zero-current potentials were -0.36 ± 0.02 V at pH 9, which is in good agreement with the formal potential of the coenzyme at this pH. During our electrochemical studies, of BV²⁺ or rosininduline 2G and NADH on D.M.E. and R.P.D.E. we observed that reaction is hardly noticeable unless an enzyme catalyst is present. Such a catalyst is not a specific dehydrogenase like GPDH but a complex oxido-reductase namely XO. Indeed, XO is a model for the whole electron-transfer chain: it contains a molybdenum, a flavin and two iron-sulphur redox-active groups⁴⁰ and may use, as electron acceptors, oxygen or dyes and, as electron donors, pyrimidines, purines, aldehydes or NADH instead of xanthine. Likewise ITO and KUWANA³⁹ detected a chemical reaction between the methyl viologen free radical MV^{•+} and NADP⁺ only when spinach ferredoxin-NADP-reductase is present in the solution.

As benzyl-viologen seems to be able to reduce or oxidize the coenzyme in the presence of XO, we established that BV²⁺ is a suitable mediator for the NAD⁺-NADH system, according to the criteria presented by WILSON:⁴¹

1. BV²⁺ interacts readily with a platinum electrode, giving an electrochemical reversible BV²⁺-BV^{•+} system, as shown on voltammetric curves on R.P.D.E. (Fig. 7). BV²⁺ gives an electrochemical reversible system also on D.M.E., but the occurrence of a strong adsorption of BV^{•+} or BV⁰ shifts its zero-current potential to a -0.18 ± 0.05 V value.

2. The formal potential of BV²⁺-BV^{•+} *i.e.* -0.359 V is close enough to the thermodynamical potential of the NAD⁺-NADH system.

3. At a concentration reaching 20 μ M BV²⁺ does not interact with the oxidized and reduced coenzyme, as it has been demonstrated by the absence of modification of 0.5 mM NAD⁺ or NADH electrochemical patterns on D.M.E. and R.P.D.E.

4. XO, the presence of which is necessary for the establishment of the equilibrium between BV²⁺ and the coenzyme, does not modify the electrochemical patterns of NAD⁺, NADH, BV²⁺ and BV^{•+}.

To conclude, in the presence of XO, BV²⁺ is a quite appropriate mediator in order to supply a reliable value of the formal potential of the NAD⁺-NADH system. The calculated values of this potential, either from zero-current potentiometric measurements on platinum and D.M.E. or from voltammetric curves on R.P.D.E. and D.M.E., are indeed in good agreement with the usually accepted -0.320 V value at pH 7. Electrodes equilibrate with BV²⁺-BV^{•+} except when the mediator is very diluted and when its adsorption equilibrium on mercury surface is not reached within the drop-time. Nevertheless, we have not succeeded in modifying the electrochemical patterns of the pyridine coenzymes and in making them to behave reversibly on an electrode, even with specific dehydrogenases and substrates.

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